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# The role of amylin receptor components in body weight and food intake control in obese knockout mouse models

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**Summary**

Zusammenfassung (Englisch)
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The role of amylin receptor components in body weight and food intake control in obese knockout mouse models

Obesity currently affects over one third of the entire world's population. The treatment of obesity and overweight is one of the most challenging topics in medicine and numerous approaches can be used to fight this expanding problematic.

The aim of this study was to assess the weight loss efficacy of the amylin selective agonist (NNCO1741213 abbreviated to NN1213) in the three RAMP KO mouse models, RAMP 1/3 RAMP 3 and RAMP 1 WT/KO, compared to that of salmon calcitonin (sCT)

Our results show that RAMP 1 or RAMP 3 are necessary for the action of NN1213 in mice. Both receptor isoforms mediate the action of amylin selective agonists on body weight and food intake. In fact, we saw a positive effect of NN1213 to lower body weight in all WT mice and in the mice, where at least one RAMP was expressed. NN1213 decreased food intake and body weight in these mice. Further, we provided evidence that subchronic sCT treatment in mice decreases body weight and food intake only in the absence of RAMP 3. In addition to that, our results suggest that subchronic sCT does not have any effect on food intake and body weight in RAMP WT mice.

Keywords

1. Amylin
2. Body weight
3. Food intale
4. Mice

**Zusammenfassung**

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Die Rolle von Amylinrezeptoren in der Kontrolle des Körpergewicht und der Nahrungsaufnahme in adipösen Knockout-Mausmodellen

Ziel der Arbeit war es, die Effekte des Amylin-selektiven Agonisten NNCO1741213 (NN1213) auf das Körpergewicht und auf die Futteraufnahme während einer 21 Tage langen Therapie von WT und RAMP 1/3; RAMP 3 und RAMP 1 KO Mäusen im Vergleich zu den Effekten von Salmon Calcitonin (sCT) zu untersuchen. Zusätzlich wollten wir die Rolle der verschiedene RAMPs in der Pharmakologie des Amylin-selektiven Agonisten (NN1213) und des Dual-amylin-calcitonin-receptor Agonist (auch DACRA genannt) sCT analysieren.
Wir konnten zeigen, dass RAMP 1 oder RAMP 3 für die Wirkung von NN1213 eine Rolle spielt. Beide Rezeptor-Isoformen vermitteln die Wirkung des Amylin Agonisten NN1213 auf das Körpergewicht und auf die Futteraufnahme. NN1213 hatte einen positiven Effekt auf das Körpergewicht bei allen WT Mäusen und bei Mäusen, bei denen zumindest einer der beiden RAMPs exprimiert war.
Zusätzlich lieferten wir den Hinweis darauf, dass eine subchronische sCT Verabreichung bei Mäusen nur in Abwesenheit von RAMP 3 wirkt. Ergänzend dazu deuten unsere Resultate darauf hin, dass sCT keinen Effekt bei RAMP WT Mäusen hat.

Schlüsselwörter

- Amylin
- Körpergewicht
- Futteraufnahme
- Mäusen

# 1. Introduction

## 1.1. Definition of obesity

The World Health Organization defines obesity and overweight as abnormal or excessive fat accumulation relative to lean mass. To assess body condition of a person, body mass index (BMI) is usually used; the BMI is the weight in kilograms divided by the square of his/her height in meters ($kg/m^2$). An adult is considered as overweight with a BMI greater or equal to 25 $kg/m^2$ and as obese with a BMI greater or equal to 30 $kg/m^2$.

Obesity, along with overweight, currently affects over one third of the entire world's population. While the prevalence of obesity in adults seems to be stable in developed countries, the prevalence of global childhood obesity and the prevalence of adult obesity in developing countries is constantly increasing[1].

Obesity per se is the result of an imbalance between calories consumed and calories expended[1], this difference creates a positive energy balance which results in excess in body weight.

The etiology of obesity seems to be a complexity of genetics, environmental, individual and sociocultural factors[1,2].

Obesity and overweight may lead to several health problems. In fact, they are risks factor for a number of chronic diseases, such as diabetes mellitus, cardiovascular disease, cancer and psychiatric disorders[1].

The treatment of obesity and overweight is one of the most challenging topics in medicine and numerous approaches can be used to fight this expanding problematic. However, many of the possibilities to manage this worldwide disease seem to fail. Changes in lifestyle, such as consuming less calories and increasing physical activity to burn more calories represent the most obvious and least invasive ways to decrease body weight[3]. Since changes in lifestyle are often neither acceptable nor satisfactory in the longer term, new alternatives to reduce body weight such as combination of hormones therapy or bariatric surgery intervention have been developed. However, bariatric surgery is invasive, expensive and reserved for patients that are critically obese. Unfortunately, most of the pharmacological treatment that were developed until recently are not satisfying in the long-term weight loss. Thus, new therapies need to be developed approaches, in terms of understanding the peripheral and central control of food intake and that are able to elicit body weight loss[3].

## 1.2. Control of food intake

A complex system of central and peripheral signaling[4] is involved in the physiological control of food intake. Of predominant interest are peripherally produced hormones, which are considered physiological controls of eating. Two groups can be distinguished: the first one includes satiation signals, considered "short-term" signals, such as cholecystokinin (CKK), amylin, peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) secreted during a meal by the pancreas and intestine[5]. They stimulate the hindbrain directly or indirectly via sensory neuronal pathways[6] and thus regulate the two meal-related processes: satiation and satiety (Figure 1).

The second group comprises adiposity signals that reflect the body's fat stores. The circulating levels of these hormones, which include insulin[7] and amylin[8], secreted by the pancreatic β-cells, and leptin[9] secreted by the adipocytes proportionally to the amount of fat mass. Together, they control long-term energy homeostasis. Leptin is mainly secreted from subcutaneous fat tissues[10], while insulin secretion is mostly related to the amount in visceral fat[10] (Figure 1).

These peptides act on the brain to control food intake and different aspects of energy metabolism.

This thesis will focus on the role of amylin in particular and its different functions are described below (see paragraph 1.4).

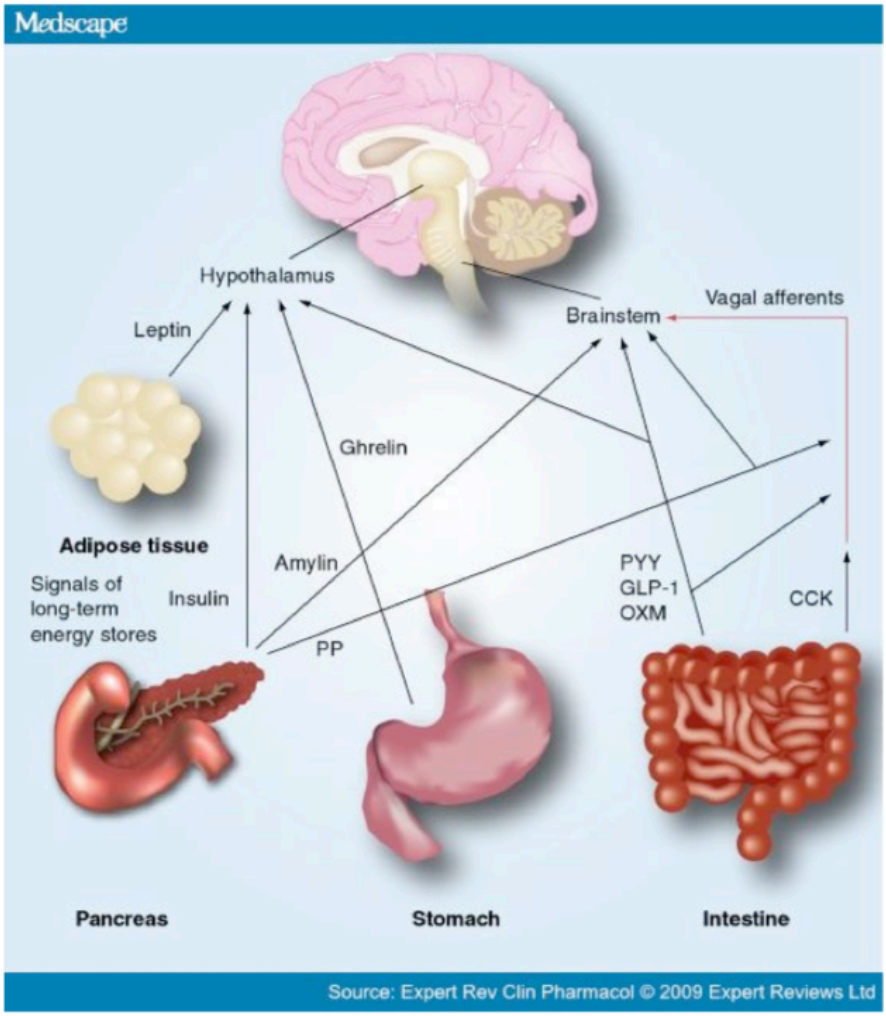


**Figure 1**: Interaction of different hormones affecting peripheral and central signaling to control food intake[11]. Abbreviations: PP, pancreatic polypeptide; PYY, Peptid YY; GLP-1, Glucagon-like Peptide 1; OXM, Oxyntomodulin; CKK, Cholecystokinin

### 1.3. Control of energy homeostasis and energy metabolism

Body fat mass and body composition reflect three processes of energy homeostasis: energy intake, energy storage and energy expenditure. Energy homeostasis is achieved when caloric intake equals energy use and dissipation, and this dynamic balance maintains body weight constant over long period of time. A chronic imbalance between energy expenditure and caloric intake can result in a surplus of energy, which will be stored as body fat mass, or lead to body weight loss, when energy consumption exceeds energy intake.

Neuronal, endocrine and metabolic signals are involved in the control of energy homeostasis and thus in the development of obesity[12]. The hormones and peptides that control meal-to-meal food intake (satiation or satiety signals) and adiposity signals play the crucial role in the

control of food intake and thus in the regulation of energy homeostasis[6]. These hormones and particularly amylin, once secreted, are transported from the periphery via the blood to activate specific brain areas. Amylin activates neurons located in circumventricular organs such as the area postrema (AP) which does not have a blood brain barrier[13] in order to control meal onset and meal size[13].

In rodents, as in humans, the energy expenditure is in part regulated through the thermogenic activity of the brown adipose tissue (BAT)[14], which is at the same time controlled by the sympathetic nervous system[14]. Recent studies indicate that amylin is able to increase BAT activity. In fact, mice that overexpress human RAMP1 (receptor activity modifying protein-1, a key component of functional amylin receptor) in neurons are lighter, have less fat and increased energy expenditure compared to the control animals[15]. Amylin is also responsible for increasing the sympathetic nerve activity, thus enhancing energy expenditure by the BAT[16]. Further, a previous study demonstrated that administration of a long actin selective amylin agonist such as sCT stimulated energy expenditure in rats[17].

## 1.4. Amylin

### 1.4.1. Amylin secretion and function

Amylin is a centrally acting 37-amino acid hormone mainly co-produced and co-secreted with insulin by beta cells of the pancreas when triggered by nutrient influx in the gastrointestinal tract[18]. Amylin acts in the brain and its main site of action is located in the AP of the caudal hindbrain[19].

Amylin has physiologically important and complementary roles with insulin in the regulation of nutrient fluxes[20]. Indeed, amylin suppresses glucagon release from the pancreas, inhibits gastric acid secretion and slows gastric emptying[21]. Amylin plays a role in the control of meal size: it reduces eating by inducing satiation, which is the promotion of meal-ending processes (Figure 2). Thus, in response to nutrient consumption, circulating amylin concentration in rats rises rapidly from a range of 3-5 pM in the fasting state to postprandial concentration of 15-25 pM[14] and it has been shown that administration of exogenous amylin peripherally reduces eating and meal size within minutes[14], while having no effect on the subsequent inter-meal interval[22]. In a pharmacological use, the desired effect of amylin's action is to decrease blood glucose, associated with long-term loss of body weight[14]. It is important to note, that amylin

and its analogs are able to reduce meal size inducing satiation, without producing any signs of conditioned taste aversion or visceral illness[14].

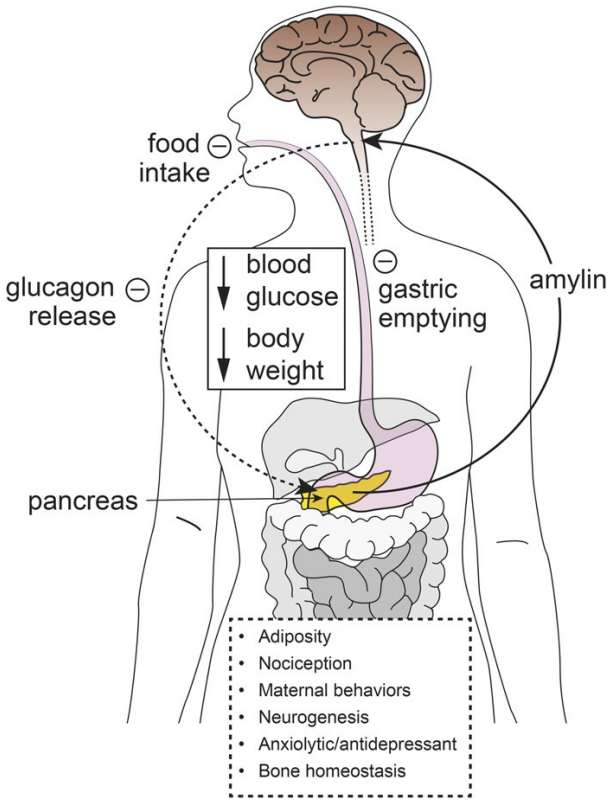


**Figure 2**. Overview of the major action of amylin[14].

### 1.4.2. Amylin as adiposity signal

Adiposity signals are hormones that are secreted in proportion to body fat[23]. As mentioned above, increased blood levels of these hormones are suggestive of a higher amount of body fat[13] and are thought to affect eating processes by enhancing the effects of satiation signals[14]. In fact, an increase in basal insulin or leptin suggests a gain in body weight which subsequently induces a lower consumption of food to restore a lower body weight at least in insulin and leptin-sensitive individual[24].

Chronic administration of adiposity signals in normal-weight individuals causes decrease in food intake and loss of body fat, while obese individuals are often resistant to these signals. Thus, while the obese state is characterized by hyperinsulinemia and hyperleptinemia[13], it is also characterized by insulin and leptin resistance[13], which challenges their therapeutic

usefulness. Like insulin and leptin, a similar role is played by amylin with basal amylin levels being directly proportional to body fat[25]. Indeed, elevated levels of fasting amylin are found in obese individuals compared to normal-weight individuals[26, 25]. It has been shown that chronic peripheral[27] or central[28] amylin administration decreases food intake and body weight, resulting in a reduction of fat depots[29]. More specifically, previous studies proved that peripherally administered amylin is able to induce body weight loss, reducing food intake and enhancing energy expenditure in obese rodents[8,30]. Additionally, chronic administration of amylin antagonists increases food intake[31] and consequently increasing body weight and body fat[28].

### 1.4.3. Amylin receptors

The peptides calcitonin (CT), calcitonin gene related peptide (CGRP), adrenomedullin (AM), amylin and adrenomedullin 2/intermedin (AM2/IMD) set up a family of closely related peptides[32]. In terms of amino acid sequence, amylin is most closely related to calcitonin gene related peptide (CGRP)[14]. In all peptides, key structural features are shared and conserved, including a N-terminal ring structure formed by a di-sulfide bond a C-terminal amide[33].

Their receptors comprise a CT receptor (CTR) or a calcitonin-like (CL) receptor (CLR) core combined with one of the three receptor activity-modifying proteins (RAMPs). The CLR by itself is not able to reach the cell surface in any remarkable amount and does not respond to any known ligand[32]. To function normally, CLR needs to bind either to RAMP1, RAMP2 or RAMP3. With RAMP1 it becomes CGRP receptor; associated with RAMP2 and RAMP3 it converts to $AM_1$ (CLR/RAMP2) and $AM_2$ (CLR/RAMP3)[32] (Figure 3).

The CTR by itself shows high affinity to CT; but when associated with one of the three RAMPs, giving $AMY_1$, $AMY_2$ and $AMY_3$ receptors[32], these receptors have a higher affinity to amylin[33,14] (Figure 3). Differently from CLR, CTR itself can reach the cell surface and be expressed, so it is likely that mixed populations of CTR/RAMP complexes and CTR alone will be found in the different brain expression sites. This makes it very arduous to clarify the action of specific CT and AMY receptors[32]. Additionally, it is important to say that no receptor subtype specific antagonists are available and that the complex between CTR and RAMP2 is extremely difficult to observe since RAMP2 KO is a lethal mouse model and therefore the pharmacology of $AMY_2$ is poorly investigated[32].

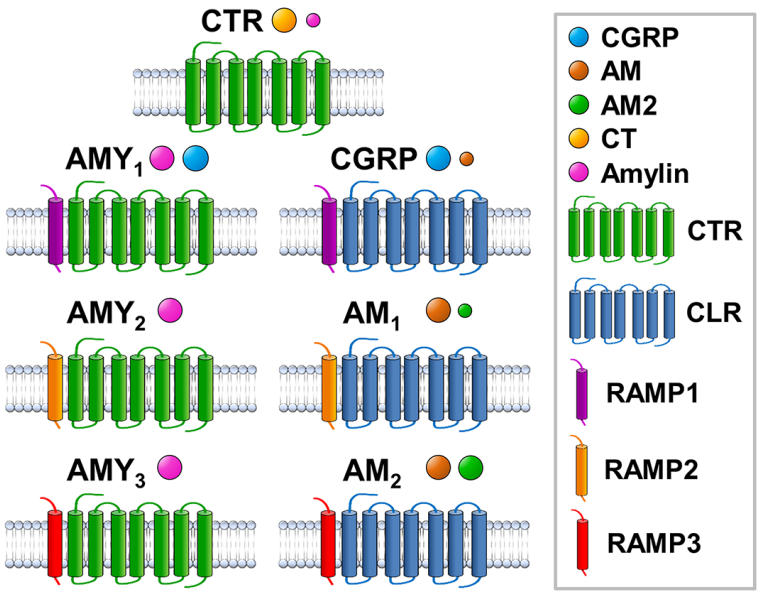


**Figure 3.** Composition and classification of human calcitonin-family receptors.
The legend in the box shows core protein receptors, ligands and receptor modifying proteins. Ligands are illustrates as spheres; relative sizes reflect the corresponding potency at each receptors; smaller sphere indicates lower potency and longer sphere indicates higher potency of a given ligand[32].

### 1.4.4. Brain expression of amylin receptor and amylin brain signaling

CTR and RAMPs are expressed in the area postrema (AP), in the nucleus of the solitary tract (NTS), in the lateral hypothalamic area, in the ventromedial (VMH) and arcuate (ARC) hypothalamic nuclei and in the ventral tegmental area[34]. Several studies have demonstrated that amylin's action depends mainly and directly on an activation of the AP, which contains a high density of amylin receptors[35, 20 36, 37,38] including all the components such as CTR, RAMP1 and RAMP3[39, 40]; the direct stimulation of AP neurons is possible because of a lack of a functional blood-brain barrier in this area[41]. To investigate the specific intracellular signaling mechanism of amylin, neuronal activation markers such as c-Fos are used. c-Fos is an early gene product which is expressed in brain areas activated by amylin and detected with immunohistochemistry[42, 38, 43, 44]. With this neuronal marker, it is possible to detect also secondary brain areas stimulated by amylin, including the NTS and the lateral parabrachial nucleus (LPB), which then transfer the neural signals to other hypothalamic nuclei[14] (Figure 4). Despite the widespread use of c-Fos, it is important to say that the exact role of the latter for amylin's behavioral effects is still unclear: it is only a marker of activation of different

brain areas. Therefore the precise signaling pathway of amylin that is necessary for its eating inhibitory effect is still under investigation[22].

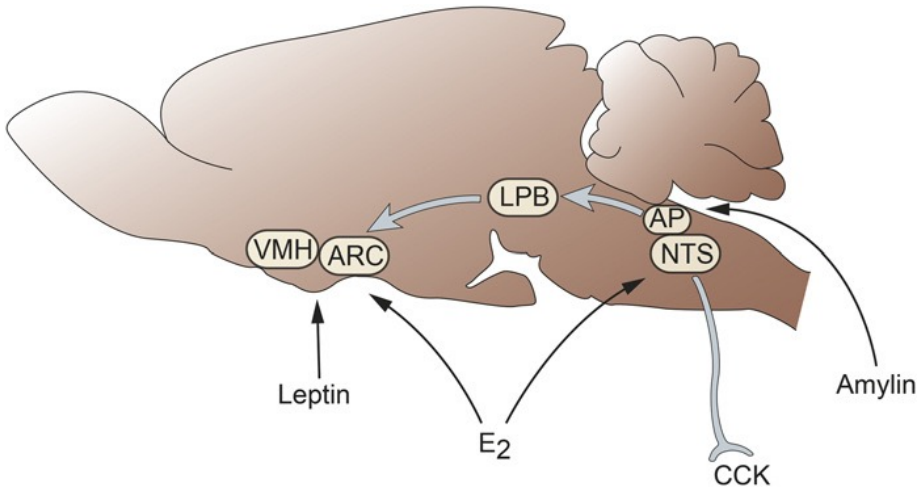


**Figure 4.** Overview of the neuronal signaling induced by amylin and its interaction with other hormones. Amylin reaches the AP via the blood circulation and activates AP neurons that project to area such as NTS and LPB[14]. Abbreviations VMH, ventromedial nucleus of the hypothalamus; ARC, arcuate nucleus; $E_2$, estradiol

### 1.4.5. Amylin agonist, obesity and diabetes: possible clinical targets

Amylin receptors are clinical targets for diabetes and obesity[45]. Many studies have assessed the effect of pramlinitide, a soluble analog of human amylin, that is currently the only approved amylin based drug (Symlin $^{R}$) to treat diabetes type 1 and type 2. Indeed these studies have shown that amylin analogues could also be useful for the treatment of obesity, especially in combination with other agents[46,47] such as metformin by decreasing food intake, meal size and eliciting a fat-specific reduction in body weight[46]. Amylin combined with leptin seems to be the most promising and clinically relevant therapeutic combination for obesity. In fact, amylin can improve leptin sensitivity and leptin resistance in obesity can be overridden by amylin[48]. However, new amylin receptor agonists with improved potency and pharmacokinetics may provide better therapeutics for the treatment of overweight and obesity.

Davalintide, an amylinomimetic with increased potency, efficacy and duration of action seems to exhibit more potency at the CGRP receptor than amylin, but binds similarly to amylin receptor as amylin does. Davalintide shows more potency than amylin in reducing food intake and body weight in rats[49]. Thus, davalintide shares similarity in the

pharmacokinetics with salmon calcitonin (sCT) (see below). However, its mode of action appears to be similar to amylin: both are able to reduce intake of palatable diet and both peptides seem to act via the AP[49, 19].

sCT is a natural dual amylin-calcitonin receptor agonist known as DACRA. It is able to activate both AMY and CT receptors[19], but not CGRP receptors[50]. Compared to amylin, sCT has a much longer duration of action, which leads to prolonged effects. Different studies have shown that sCT may be a potential treatment for some metabolic disorders: in fact, it elicits body weight loss, elevates energy expenditure, controls food intake and improves glucose metabolism in rats[51, 52, 53, 54].

Based on these findings, several dual amylin and calcitonin receptors agonist have been described. The most investigated compounds, known by "KBP" codes, are KBP-042, KBP-088 and KBP-089[50, 55, 56]. These compounds present the potency of sCT, whilst increasing their tolerability in rats[56]. The pharmacology of these molecules, at the level of the receptor, has not been extensively studied yet: it has only been shown that they stimulate, similar to sCT, both $AMY_3$ and CT receptors, but not CGRP receptors[32]. Furthermore, the pharmacology at other AMY receptors is not yet characterized; this makes it difficult to understand their receptor potency and their mechanism of action. Additional analyses are required to validate these DACRA compounds and assess their activity at different receptor complexes[32].

## 2. Aims and hypotheses

The primary focus of our research was to investigate the role of RAMPs in modulating the pharmacological effects and potential differences of the dual amylin and calcitonin receptor agonists sCT in comparison to an amylin selective analogue, NN1213, on food intake and body weight. To assess these effects, single and double RAMP KO mice models were used.
It is important to note that most published data on the anti-obesity effects of amylin and calcitonin agonist are based on rat models and not on mice models. Published data from Novo Nordisk indicate that diet-induced obese mice respond differently to both amylin and sCT analogues than obese rats. Indeed, mice seem to be resistant to the effect of subchronic sCT on food intake and body weight while rats are not. Hence, using RAMP KO mice models, we aimed at understanding these differences.

Novo Nordisk synthetized and characterized a long actin amylin selective analogue (NN1213), which will be more suitable to use in comparison to sCT.

**Hypothesis:**
The amylin-selective agonist NN1213 acts via RAMP 1 and/or RAMP 3 to elicit weight loss while sCT can elicit weight loss in the absence of RAMP 1 and or RAMP 3 in mice.

## 3. Material and methods

### 3.1. Mice and Husbandry

RAMP 1 KO, RAMP 3 KO and RAMP 1/3 double knockout (KO) mice on a 129s2/SvEv background were kindly provided by Kathleen Caron of the Department of Cell Biology and Physiology, University of North Carolina, USA.
Mice imported from the USA were rederived by the Institute of Laboratory Animal Science by embryo transfer for hygiene reason and transferred to the LASC-NGZ facility.
RAMP1/3 KO were maintained on a KO breeding scheme while RAMP 1KO and 3KO were bred my mating heterozygous mice. RAMP 3 WT mice also served as WT controls for the RAMP 1/3 KO mice.

Male mice were housed in an environment maintained at 21 ±2°C, under a 12/12 hour light-dark cycle (lights off at 19.00 h). Mice had *ad libitum* access to standard chow (Kliba 3436, 3.14 kcal/g of food) and water, until the age of approximately 4 weeks. Then mice were maintained on 45% fat high fat diet (HFD, D12451 Research Diet, 4.73 kcal/g of food, New Brunswick, NJ, USA) *ad libitum* from approximately 4 to 25 weeks of age to induce obesity. The same diet was then continued during the 21-day treatment period. The mice were group housed (2/3 mice per cage) until 12 week of age, when they were single housed into T2L cages due to aggressive behavior. Each cage was furnished with a red plastic house and nest-building material and wood shaving.

All procedures involving animals and their care were approved by the Veterinary Office of the Canton of Zurich, Switzerland, and in accordance with the EU Directive on the protection of animals used for scientific purposes.

### 3.2. Experimental diet

For the entire experiment, mice were maintained on a 45% HFD (Diet D12451, Research diets Inc., New Brunswick, NJ, USA).

Table 1: 45% HFD composition in percent of total energy content

| Product | kcal% |
|---|---|
| Protein | 20 |
| Carbohydrates | 35 |
| Fat | 45 |
| | 4.73 kcal/gram |

### 3.3. Genotyping

#### 3.3.1. RAMP 3 WT and KO mice genotyping

RAMP 3 WT and KO mice were genotyped at the age of 3-4 weeks by PCR analysis of toe DNA. Briefly, 200 µl lysis buffer (50 mM NaOH) were added per biopsy and every tube was incubated at 95° C and shaken at 800 rpm for 35 min. After that, 200 µl of neutralization buffer (500 mM Tris-HCL) was added per biopsy and underwent PCR.

Genotype was determined using the following primers (Microsynth AG, Balgach, Switzerland):

- RAMP3 R3-1- 5'-GTGCTCAAGGGTTCTGTCTG-'3,
- RAMP3 R3-10-5'-GACCTGGTTCATCTCTGGCTC-3'
- RAMP3 Neo6-10- 5'-GCTTCCTCTTGCAAAACCACA-3'

We performed two different PCRs: one for the WT band detection and one for the KO band detection. For the WT PCR, 1 µl DNA of every biopsy was used and to each of them, we added 15.85 µl $H_2O$, 5 µl green PCR buffer, 1 µl dNTPs, 1 µl R3-1, 1 µl R3-10 and 0.15 µl Go-Taq 5 u/µl mixed all in a same mastermix reaction. The protocol used for the WT PCR was the following: first cycle of 5 minutes at 95°C, after that 40 x cycles of 30 seconds at 95°C, 30 seconds at 59°C and 90 seconds at 72°C and after that one last cycle of 10 minutes at 72°C.

For the KO PCR we used 1 µl DNA of every biopsy and to each of them we added 15.85 µl $H_2O$, 5 µl green PCR buffer, 1 µl dNTPs, 1 µl neo-60, 1 µl R3-10 and 0.15 µl l Go-Taq 5 u/µl mixed all in a same mastermix reaction. The protocol used for the KO PCR was the

following: a cycle of 5 minutes at 95°C, after that 40 x cycles of 30 seconds at 95°C, 30 seconds at 56°C and 90 seconds at 72°C and after that one last cycle of 10 minutes at 72°C.
To detect and separate the PCR products, 100 ml 2% agarose gel with 4 ul gel red was prepared and for every sample 6 μl of WT reaction mix and 6 μl KO reaction mix were put side-by-side and gel electrophoresis was run for 40 min at 100V. Finally, the gel was photographed with Biorad Reader and the genotype was determined. The WT PCR produced a band of 650 bp while the KO PCR produced a 675 bp band.

### 3.3.2. RAMP 1 WT and KO mice genotyping

RAMP 1 WT and KO mice were genotyped at the age of 3-4 weeks by PCR analysis of toe DNA. 300 μl isolation buffer and 3.5 μl Proteinase K were added to mouse tissue in a microtube. The tubes were incubated at 95°C until tissue was dissolved. 600 μl of cold 100% ethanol were added per tube and the samples were then centrifuged at 14000 rpm for at least 2 hours, the supernatants were poured off. The same was repeated for 30 seconds. Then the samples were dried at air for 10 minutes and resuspended in 100 μl of TE buffer or water.
We performed one single PCR using the following primers:

- Forward Primer: TCATGGGGACCTTTAGGTAAGC
- Reverse Primer: ACAGCAATCCTTCTACCTCAACAC

The protocol used for RAMP 1 genotyping was the following:
PCR machine performed 1 cycle at 95°C for 5 minutes, 40 cycles at 95°C for 45 seconds, at 59°C for 45 seconds and at 72°C for 90 seconds, 1 cycle at 72°C for 10 minutes and the last cycle at 22°C until use.
We prepared 100 ml of 2% agarose gel with 4 μl gel red. 6 μl of each sample were put in the gel and gel electrophoresis was let run for 40 min at 100V. Finally, the gel was photographed with Biorad Reader and the genotype was determined. The WT PCR produced a band of 1600 bp while the KO PCR produced a 400 bp band.

## 3.4. Food intake, body weight and non-fasted blood glucose level during 20 weeks on 45% high fat diet

Weekly body weight and food intake were measured manually using a digital scale (Mettler Toledo x540025 Delta Rage scale). Blood was harvested from the tail and non-fasted glucose was measured using Contour Next Blood Glucose Meter (Bayer Consumer Care AG, Basel, Switzerland) one time every two weeks at 14.00 h, i.e. in the second half of the light phase.

### 3.5. Handling and acclimation

Prior to any testing, mice were given one week to acclimate to the daily injections to reduce stress. Mice were handled and injected subcutaneously in the neck once daily with 1 ml/kg saline solution. 1 ml sterile syringes (B. Braun Melsungen AB, Melsungen, Germany) and 26G needles were used for the injections.

### 3.6. Peptides

All in the experiment used drugs were provided from Novo Nordisk Pharma AG:

- Vehicle: Buffer 9302, pH 4.0, 5 mM acetate, 249 nM propylene glycol, 0.007% Tween 20
- NNCO1741213 (NN1213): 5 nmol/ml, 30 nmol/kg
- salmon Calcitonin (sCT) 0186: 27.7 nmol/ml, 150 nmol/kg

### 3.7. Mice chronic treatment and sacrifice

At the age of 25 weeks and after 20 weeks on 45% HFD, male RAMP 1/3 KO, RAMP 1 KO and RAMP 3 KO and their respective WT male littermates were randomized by body weight and assigned to their treatment group (n=8/group):

- WT vehicle;
- WT NN1213, dose 30 nmol/kg;
- WT sCT, dose 150 nmol/kg;
- KO vehicle;
- KO NN1213, dose 30 nmol/kg;
- KO sCT, dose 150 nmol/kg.

Mice were injected subcutaneously once a day for 21 days in the middle of the light phase between 11.00-12.00h. Prior to injection, daily body weight and food intake were measured manually using a digital scale (Mettler Toledo x540025 Delta Rage scale).

At the end of treatment, animals were fasted for 2 hours during the light phase, sacrificed by exsanguinations under deep pentobarbital anesthesia (100 mg/kg, IP injection, Cantonal Pharmacy of Zurich, Switzerland). Blood was sampled using cardiac puncture into 1 ml EDTA tubes. Brains were removed, frozen on dry ice and stored at -80°C. The mice were sutured closed after exsanguination and stored at -20°C until body composition analysis was performed on the carcasses. To have comparable results with the rest of the study, tail blood glucose level was assessed using Contour Next Blood Glucose Meter (Bayer Consumer Care AG, Basel, Switzerland).

### 3.8. Measurement of body composition

Quantitative microcomputed tomography of WT mice and RAMP 1/3 KO was performed using La Theta LCT-100A (LaTheta LCT-100A scanner, Hitachi-Aloka Medical Ltd., Tokyo, Japan) on the carcasses of the animals placed supine in the plexiglass holder after sacrifice. The X-ray source tube voltage was set at 50 kV with 1 mA current. A sagittal image of the animal was used as an overview about the position of the animal, after that scans of the entire animals were done. To avoid artifacts, the legs were adequately extended. The region between the vertebrae L1-L6 was evaluated for lean and fat mass. Although the software LaTheta already automatically distinguished between visceral and subcutaneous fat, each image was manual examined and in some cases an image-by-image correction was required. Adipose tissue weights were then computed using the commonly used density factor of 0.92 $g/cm^3$ to determine the absolute values in gram[57].

### 3.9. Biochemical analysis

Prior to sampling, mice were food deprived for 2h. Blood samples were collected from the right ventricle of the heart from all animals during the sacrifice in 1ml EDTA-coated tubes containing DPP4 inhibitor (10 ul/1 ml blood) and proteinase inhibitor (P2414; Sigma Aldrich, 30ul/1 ml blood) to stop the enzymatic degradation of peptide hormones, centrifuged at 12000 rpm for 5 minutes at 4°C. Plasma was transferred to clean Eppendorf tubes and kept at -80°C

until further analysis. Plasma level of leptin, insulin, GLP-1 and glucagon were measured with 4 plex-Mouse Metabolic Biomarkers kit (MesoScale Discovery, Gaithersburg, MD, USA) according to the manufacturer's instruction.

### 3.10 Statistical analysis

All analyses were performed using GraphPad Prism software for Mac OS X version 8.0 (GraphPad, San Diego, CA). In experiment comparing independent treatment groups, significance was tested using one-way ANOVA. When more than one factor were compared, we used two-way ANOVA, followed by Sidak or Tukey's multiple comparisons test as recommended. A P-value $< 0.05$ was considered statistically significant. All data are presented as mean ±SEM.

# 4. Results

## 4.1. RAMP 1/3 wild-type and double knockout mice

### 4.1.1. Obesity induction period

Male mice were 4 weeks-old when recruited for this experiment. From 4 to 24 weeks old, mice body weight and food intake on 45% HFD was followed weekly (Figure 1).

Baseline body weight was similar across both groups with no significant difference at the start of the experiment (16-17g). During the first 7 weeks, the body weight between these two genotype groups was not significantly different. From week 8 onward, RAMP 1/3 KO mice displayed a significantly lower body weight ($p<0.05$) compared to the WT cohort and it remained significantly lower until the end (Figure 5A). At the end of the obesity induction period, average body weight was 44g in the WT mice and 33.4g in the KO mice (Figure 1A).

Although the two groups showed no significant difference in body weight gain during the first 6 weeks on 45% HFD, RAMP 1/3 WT started to gain more weight than KO after 3 weeks on 45% HFD which became significant at 11 weeks-old (Figure 5B).

Furthermore, after 9 weeks on HFD, RAMP 1/3 WT mice ate significantly more than the double KO mice. In fact, cumulative food intake measured in RAMP 1/3 WT animals remained significantly higher compared to the double KO until the end of the HFD period (Figure 5C) which is in line with the increase in body weight.

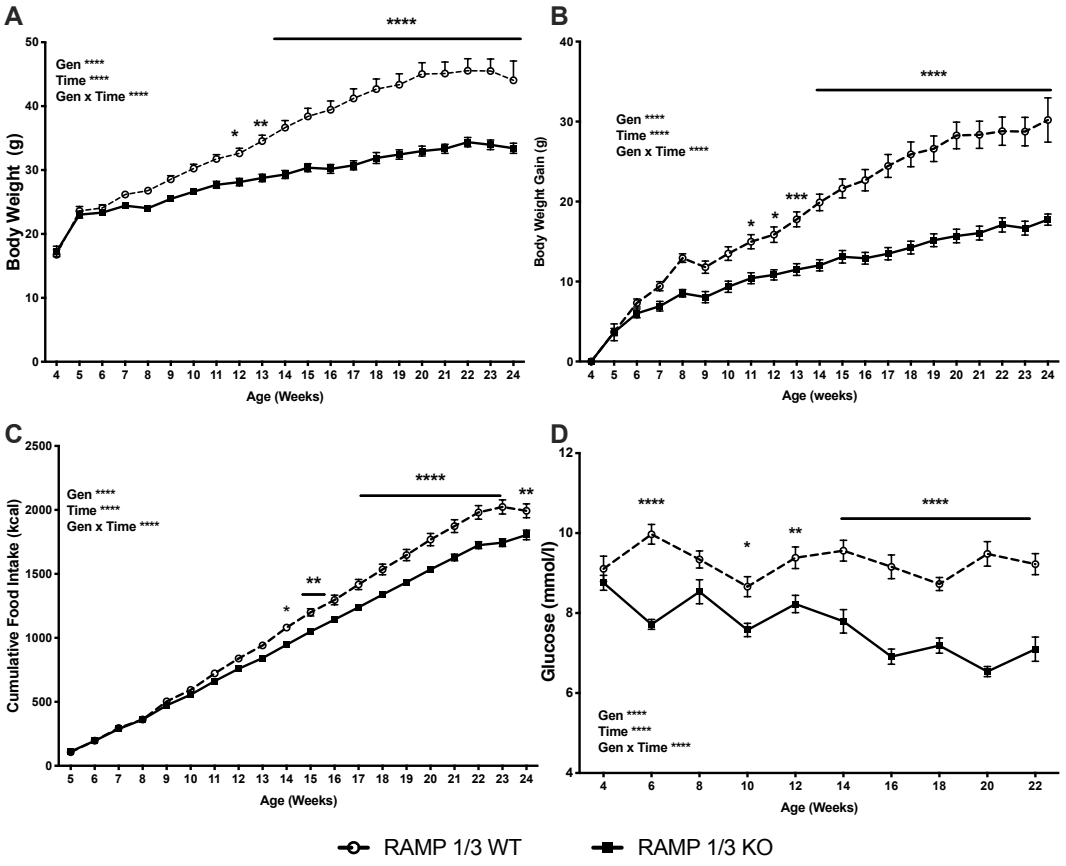


**Figure 5: Obesity induction period in RAMP 1/3 WT and KO mice fed with 45% HFD ad libitum for 20 weeks. A:** Weekly body weight (g) in RAMP 1/3 WT and KO mice over 20 weeks on 45% HFD. **B:** Weekly body weight gain (g) in RAMP 1/3 WT and KO mice over 20 weeks on 45% HFD. **C:** Weekly cumulative food intake (kcal) of RAMP 1/3 WT and KO mice over 20 weeks on 45% HFD. **D:** Non-fasting blood glucose level (mmol/l) of RAMP 1/3 WT and KO mice maintained on 45% HFD for 20 weeks measured on a bi-weekly base. All data are expressed as mean ± SEM, symbols denote significant differences between the two genotypes; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N = 24 per group.

Analysis of non-fasting tail blood glucose measured on a bi-weekly basis (Figure 5D) showed that baseline blood glucose level was similar across both groups with no significant difference before the start of the 45% HFD period.

Then, for the entire HFD period, RAMP 1/3 WT displayed a higher blood glucose level than RAMP 1/3 KO mice after 2 weeks of HFD and between weeks 12 and 22 (Figure 5D).

### 4.1.2. Effect of amylin-selective agonist NN1213 and sCT in RAMP 1/3 WT and KO mouse models on body weight, food intake and blood parameters after 21 days of treatment

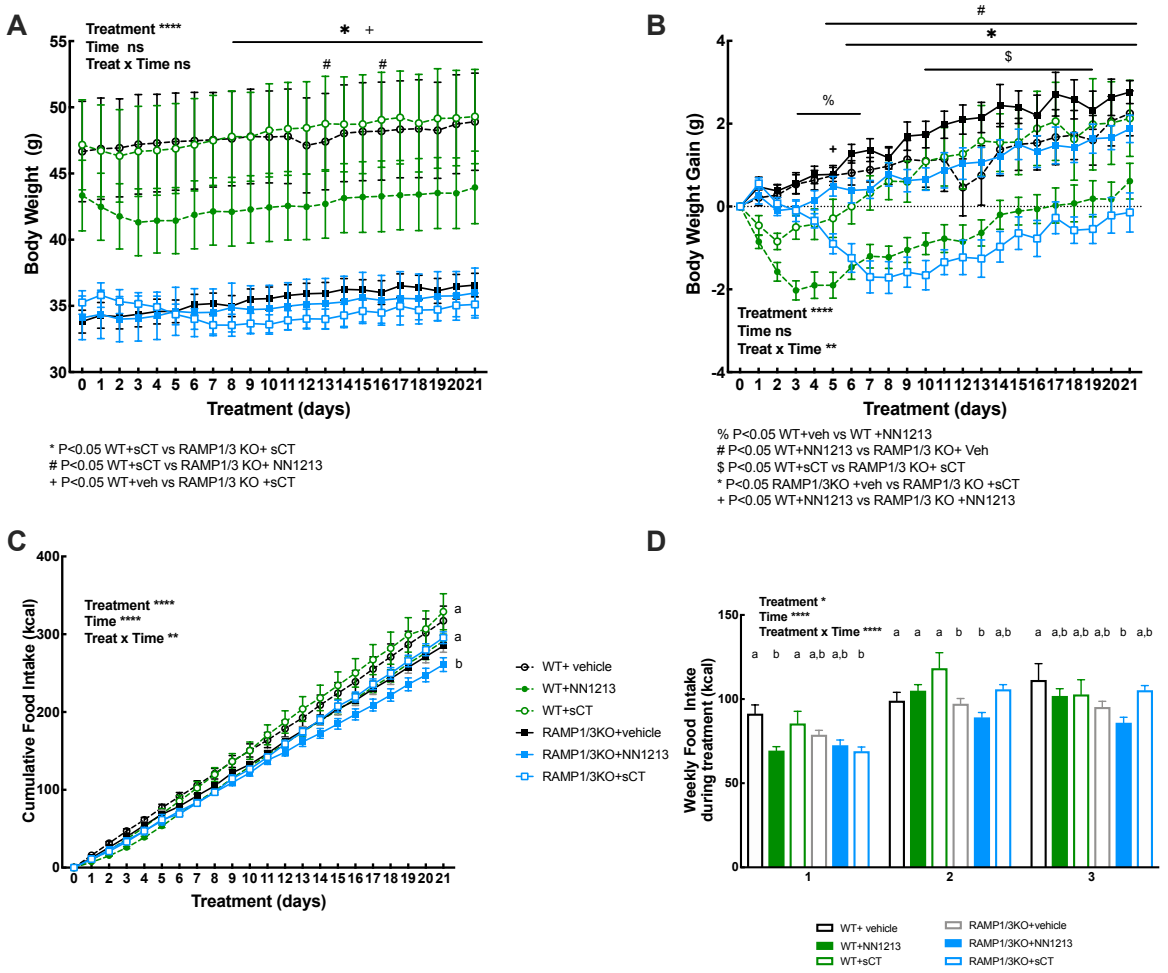


**Figure 6: Effect of amylin-selective agonist NN1213 (30 nmol/kg) and sCT (150 nmol/kg) subchronic treatment for 21 days in RAMP 1/3 WT and KO mice after an induction obesity period of 20 weeks on 45% HFD A:** daily BW (g) of RAMP 1/3 WT and KO mice treated for 21 days with vehicle, NN1213 (30 nmol/kg) and sCT (150 nmol/kg). **B:** daily body weight gain (g) of RAMP 1/3 WT and KO mice treated daily with vehicle, NN1213 and sCT for a period of 21 days. **C:** daily food intake of RAMP 1/3 WT and KO mice during 21 days of treatment with vehicle, NN1213 and sCT. Data are represented as mean ± SEM. N= 8 per group. Parameters with differing superscript letters differ from each other at p<0.05 level by Sidak post hoc adjustment after significant intergroup differences were found by two-way ANOVA. Other statistical differences are indicated under each figure.

After 20 weeks on 45% HFD, mice were randomized by body weight into 6 groups (n=8/group) and assigned to a specific treatment group.

Body weight of RAMP 1/3 WT and KO vehicle-treated groups didn't show any intra-genotype difference throughout the entire experiment and they continued to constantly gain weight. WT and double RAMP 1/3 KO animals had a significantly different baseline body weight and this difference in body weight remained significant until the end of the treatment period (Figure 2A).

From day 1 to day 8, RAMP 1/3 WT injected with the amylin-selective agonist NN1213 showed a marked weight loss ($p<0.05$, Figure 2B) and gained significantly less body weight from day 3 to day 6 in comparison to WT vehicle group ($p<0.05$, Figure 2B). After this initial significant effect, RAMP 1/3 WT NN1213-treated mice stopped responding to the treatment and regained weight until the end of treatment period. In comparison, RAMP 1/3 KO mice receiving the same dose of NN1213 lost no weight in the first treatment days and continued to gain weight throughout the treatment period (Figure 2A, B).

RAMP 1/3 WT mice treated with sCT decreased their body weight for the first 2 days, they then stop responding to the drug and slightly gained weight during the rest of the treatment period. Conversely, RAMP 1/3 KO mice treated with the same dose of sCT gained weight after 1 day of treatment but afterwards, between day 2 and day 9, they showed a gradual decrease in body weight gain. Indeed, from day 10 until the end of the experiment, a significant difference in body weight between RAMP 1/3 WT and RAMP 1/3 KO mice treated with sCT was detected (Figure 2A, B). Hence, sCT produced lasting weight loss in the RAMP 1/3 KO but not in the RAMP 1/3 WT mice.

For the entire duration of the experiment, cumulative food intake was significantly lower in RAMP 1/3 double KO mice treated with the compound NN1213 than the other groups, which ate a similar amount of food during the treatment period (Figure 6. C).

The analysis of food intake on a weekly basis (Figure 6. D) during the treatment period in the six groups of mice showed that RAMP 1/3 WT mice treated with the amylin-selective agonist NN1213 consumed a significant lower amount of food during the first week compared to the other groups, with the exception of RAMP 1/3 double KO group treated with sCT, which showed a similar behavior. We didn't notice the same effect in the second and third week.

Thus, RAMP 1/3 WT decreased their body weight gain, for the first week, when treated with NN1213 while RAMP 1/3 KO did not. Even though KO NN1213-treated RAMP 1/3 did not decrease their body weight, the treatment decreased their food intake. Further, sCT-treated WT mice responded to the treatment by increasing their body weight while RAMP1/3 KO

had the opposite effect and showed a decrease in body weight gain during the first week of treatment. However, sCT did not affect food intake in RAMP 1/3 KO.

### 4.1.3 Effect of amylin-selective agonist NN1213 and sCT in RAMP 1/3 WT and KO mice models on blood parameters and body composition

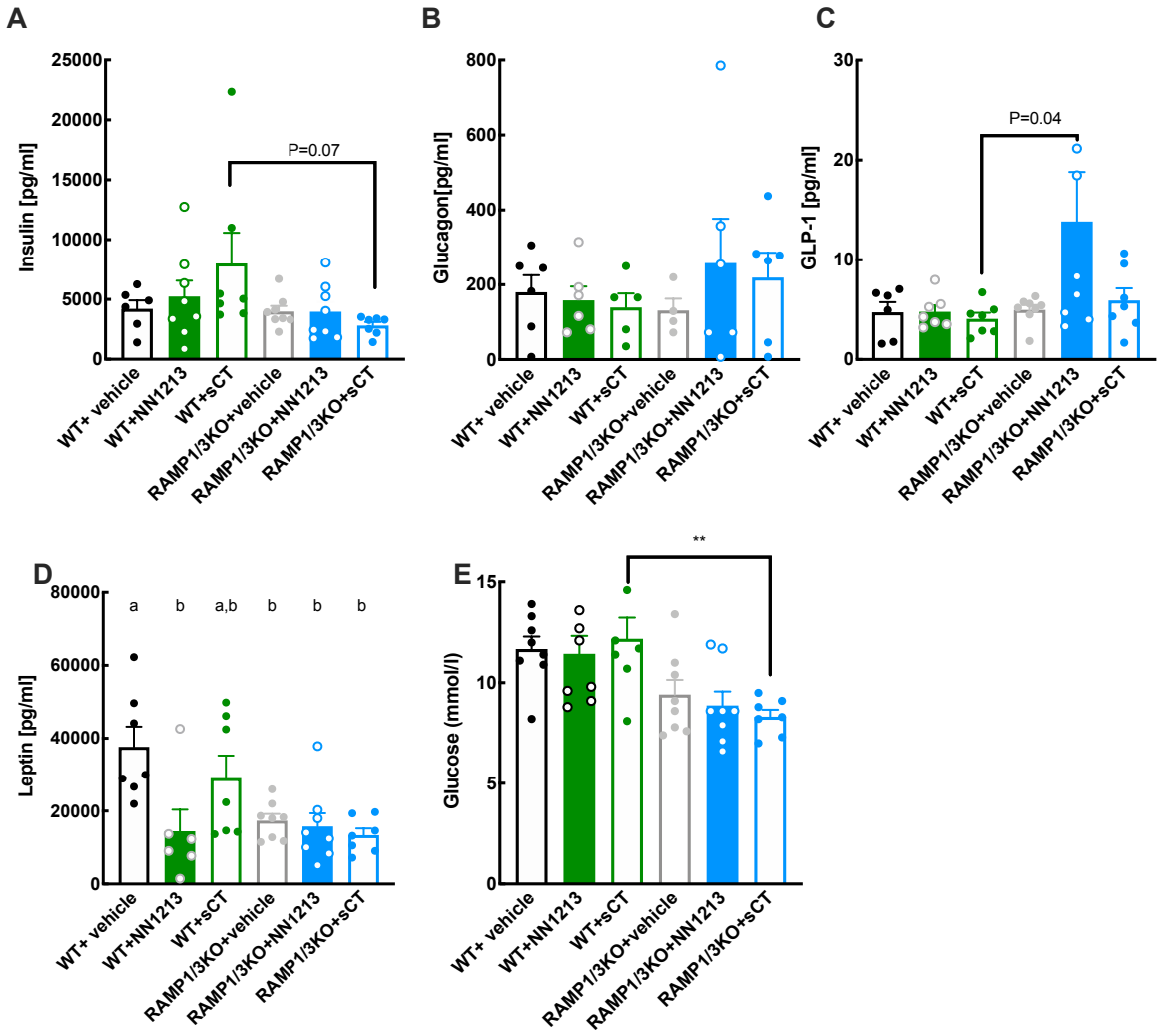


**Figure 7: Insulin, Glucagon, GLP-1, Leptin (pg/ml) and Glucose (mmol/l) concentration measured in plasma of RAMP 1/3 WT and KO mice after 21 days of treatment with vehicle, NN1213 (30 nmol/kg) or sCT (150 nmol/kg) at time of sacrifice.** All data are presented as mean ±SEM; N= 8/group. Parameters with differing superscript letters offer from each other at $p<0.05$ level by Tukey and Sidak post hoc adjustment after significant intergroup differences were found by two-way ANOVA. * Symbols denote significant differences between the two genotypes; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Insulin and glucose plasma levels were lower in RAMP1/3 KO treated with sCT than in WT sCT-treated mice (Figure 7A, E). This difference is probably result from the lower body weight seen in sCT-treated RAMP 1/3 KO mice compared to sCT-treated WT mice (Figure 6A).

Glucagon plasma concentration was not significantly different between groups (Figure 7B). However, RAMP 1/3 double KO treated with NN1213 showed significantly higher GLP-1 levels than WT treated with sCT (Figure 7C)

WT mice injected with NN1213 had a significant lower leptin concentration in plasma compared to the WT receiving vehicle. WT receiving sCT showed a higher leptin concentration in comparison to the NN1213 treated group but this difference was not statistically significant. RAMP 1/3 KO mice displayed lower leptin levels compared to WT mice treated with vehicle or sCT (Figure 7D) but were similar to WT+NN1213 mice.

To assess body composition in the different groups, lean and fat mass were measured using CT scan. The region between the vertebrae L1-L6 was evaluated. Figure 8 shows body composition (in g) of the six groups of treatment. Lean mass was significantly lower in RAMP 1/3 double KO mice compared to the WT cohort, regardless of the treatment received which reflects their lower body weight in general. The data showed a trend with a p-value of 0.06 in the visceral fat mass between RAMP 1/3 WT mice and double KO treated both with sCT.

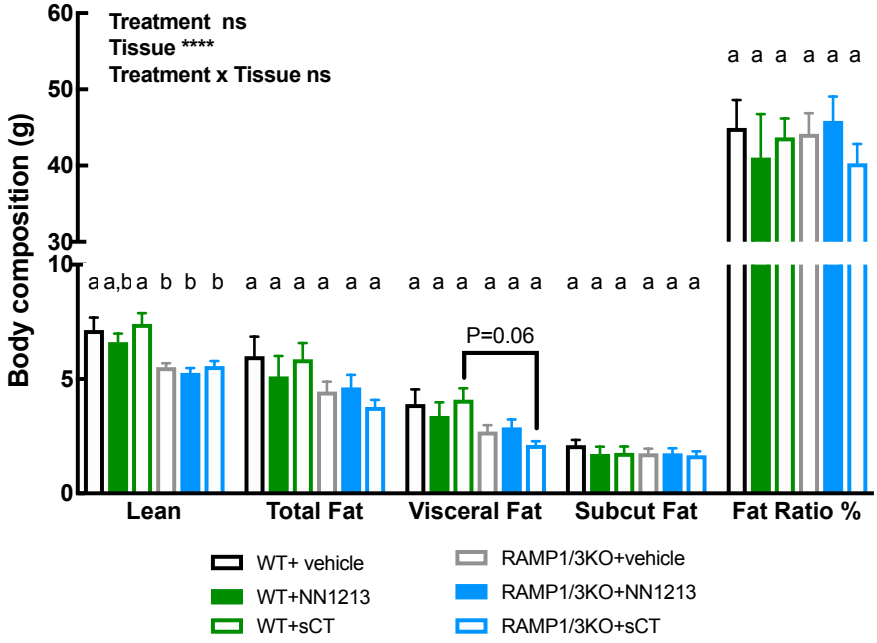


**Figure 8: mass of lean, total fat, visceral fat, subcutaneous fat in grams and fat ratio% measured by CT scan in RAMP 1/3 WT and KO injected with vehicle, NN1213 and sCT for 21 days after 20 weeks on 45% HFD.** Parameters with differing superscript letters differ from each other at $p<0.05$ level by Tukey multiple comparisons test after significant intergroup differences were found by two-way ANOVA. Data are represented as mean ± SEM; N=8/group.

## 4.2. RAMP 3 wild-type and knockout mice

### 4.2.1 Obesity induction period

RAMP 3 WT and KO mice were 5 weeks-old when put on 45% HFD ad libitum for 20 weeks.

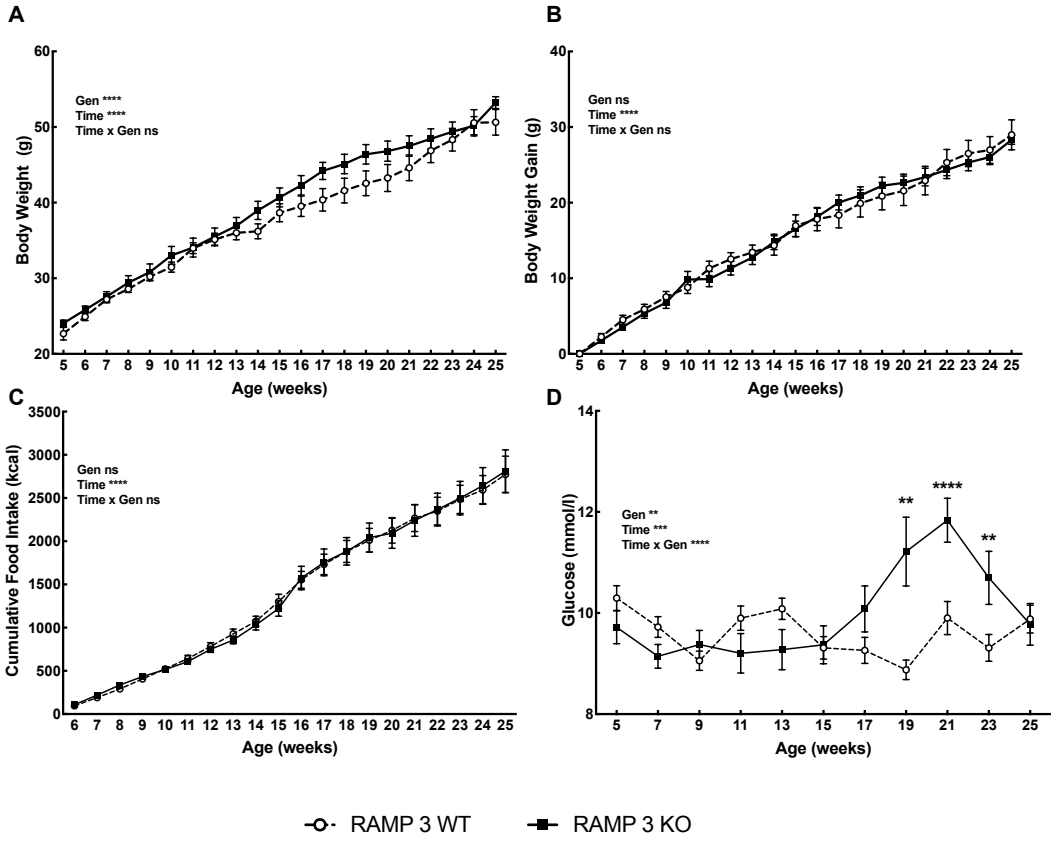


**Figure 9: Obesity induction period in RAMP 3 WT and KO mice fed with 45% HFD ad libitum for 20 weeks A:** Weekly body weight (g) in RAMP 3 WT and KO mice over 20 weeks on 45% HFD. **B:** Weekly body weight gain (g) in RAMP 3 WT and KO mice over 20 weeks on 45% HFD. **C:** Weekly cumulative food intake (kcal) of RAMP 3 WT and KO mice over 20 weeks on 45% HFD. **D:** Biweekly non-fasting blood glucose level (mmol/l) of RAMP 3 WT and KO mice maintained on 45% HFD for 20 weeks. All data are expressed as mean ± SEM, n=24/group. Symbols denote significant differences between the two genotypes; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Baseline body weight average was similar (22-24g) in both groups at the beginning of the observation time. From week 7 until week 20, RAMP 3 KO mice gained slightly more weight compared to RAMP 3 WT mice, but this difference was never significant (Figure 9A).

Body weight gain showed no significant difference between the groups throughout the HFD period. The average of body weight after 20 weeks on 45% HFD was 50.6 g and 53.2 g for RAMP 3 WT and RAMP 3 KO mice respectively (Figure 9B).
Consistent with body weight results, no significant differences were observed regarding food intake between WT and KO mice (Figure 9C).
Bi-weekly tail blood glucose average measured in the two groups of mice showed that between week 14 and week 18, RAMP 3 KO mice had a significantly higher blood glucose level compared to RAMP 3 WT mice (Figure 9D).

### 4.2.2 Effect of amylin-selective agonist NN1213 and sCT in RAMP 3 WT and KO mice models after 21 days of treatment

Baseline body weight was not significantly different at the start of the injection period (Figure 10A) and body weight remained similar among the groups during the treatment period (Figure 10A). However, from day 1 to day 5, body weight gain was decreased in RAMP 3 WT mice treated with NN1213 (Figure 10B). A marked decrease in body weight gain was also seen in RAMP 3 KO mice treated with sCT between day 1 and day 8 but this effect was lost from day 12 onwards, where a rebound effect was noticed. We didn't observe any significant difference among WT and KO treated with the amylin-selective agonist NN1213, but as seen on the graph, the drop in body weight gain was more marked, more rapid and longer lasting in the WT mice rather than in the KO mice. No significant difference between the groups in the cumulative food intake was measured throughout the entire experiment (Figure 10D).
Overall, a decreased in body weight gain in response to NN1213 was observed in RAMP 3 WT mice while RAMP 3 KO mice responded to sCT by decreasing their body weight gain (Figure 10B).

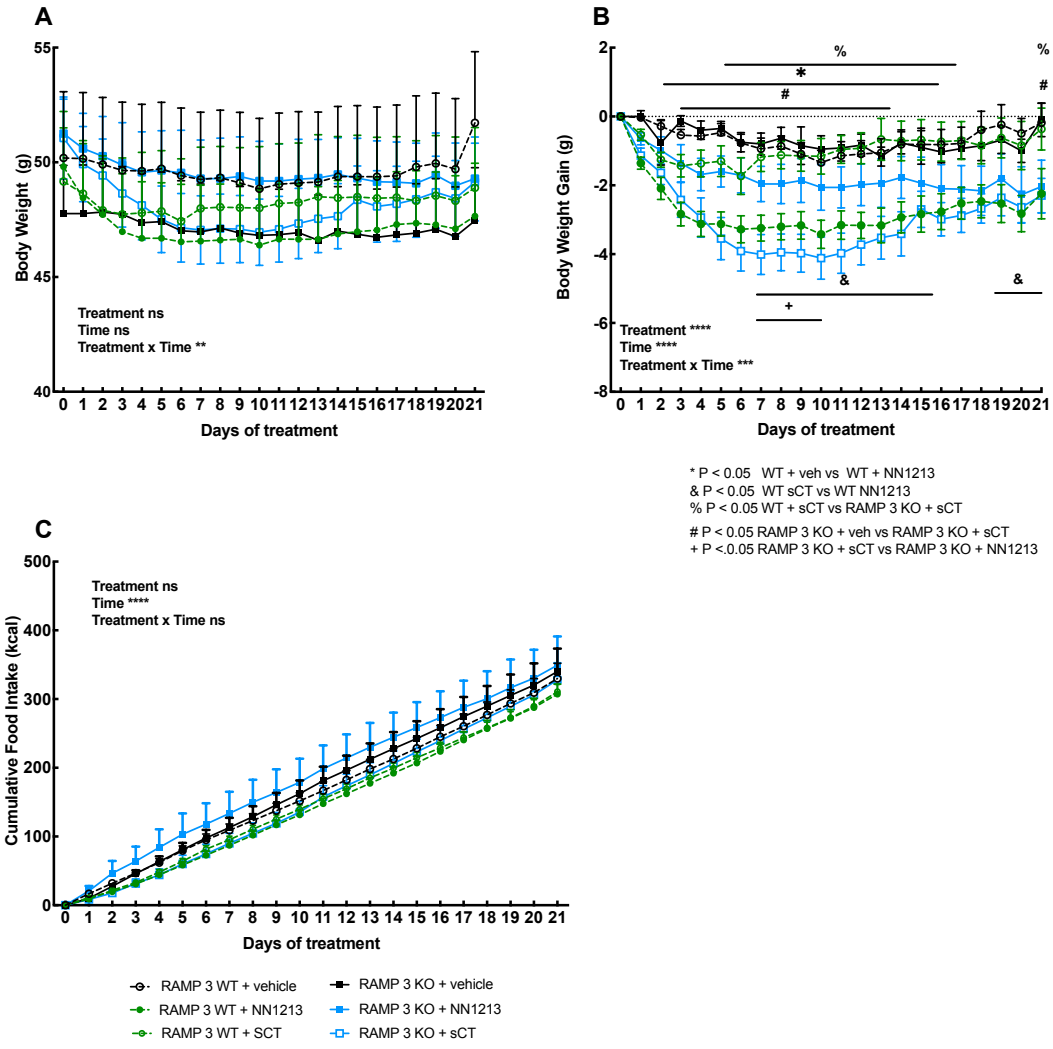


**Figure 10: Effect of amylin-selective agonist NN1213 (30 nmol/kg) and sCT (150 nmol/kg) subchronic treatment (21 days) in RAMP 3 WT and KO mice after an induction obesity period of 20 weeks on 45% HFD. A:** daily BW (g) of RAMP 3 WT and KO mice treated for 21 days with vehicle, NN1213 (30 nmol/kg) and sCT (150 nmol/kg). **B:** daily body weight gain (g) of RAMP 3 WT and KO mice treated daily with vehicle, NN1213 and sCT for a period of 21 days. **C:** daily food intake of RAMP 3 WT and KO during 21 days of treatment with vehicle, NN1213 and sCT. Mean ± SEM, N=8/group. Parameters with differing superscript letters differ from each other at $p<0.05$ level by Tukey post hoc adjustment after significant intergroup differences were found by two-way ANOVA.

### 4.2.3 Effect of amylin-selective agonist NN1213 and sCT in RAMP 3 WT and KO mice models on blood glucose level

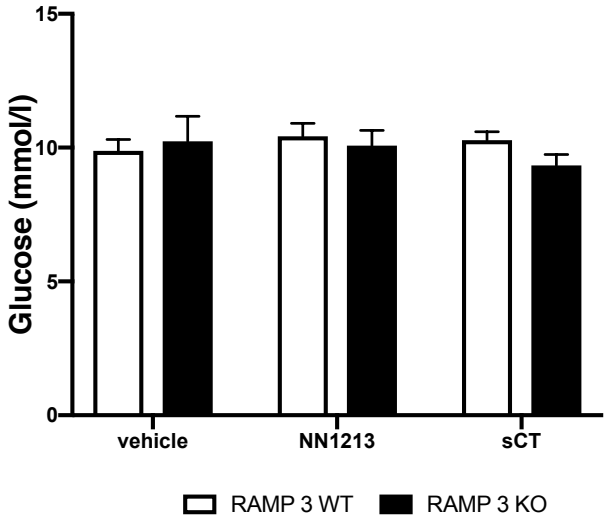


**Figure 11: blood glucose level (mmol/l) at time of sacrifice in RAMP 3 WT and KO mice after been treated daily for 21 days with vehicle, NN1213 (30 nmol/kg) and sCT (150 nmol/kg).** Data are represented as Mean ± SEM. N=8/group

At the time of sacrifice, blood glucose levels were similar regardless of genotype or treatment.

## 4.3. RAMP 1 wild-type and knockout mice

### 4.3.1 Obesity induction period

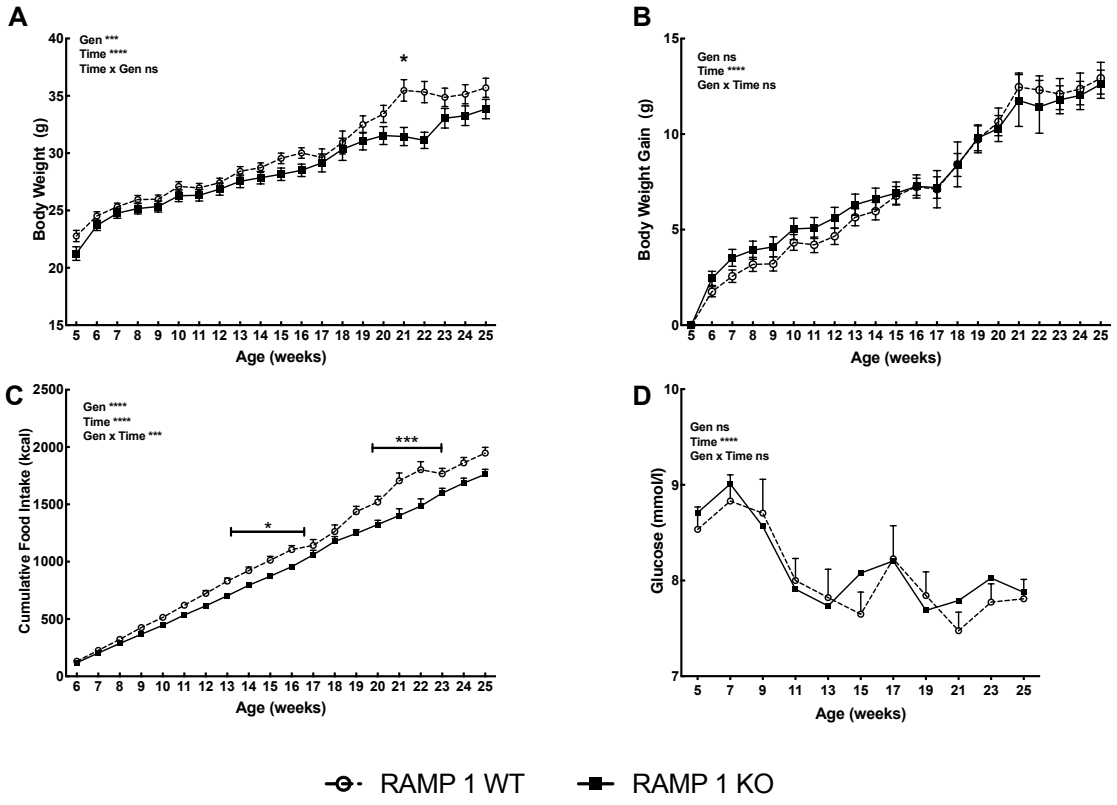


**Figure 12: Obesity induction period in RAMP 1 WT and KO mice fed with 45% HFD ad libitum for 20 weeks. A:** Weekly body weight (g) in RAMP 1 WT and KO mice over 20 weeks on 45% HFD. **B:** Weekly body weight gain (g) in RAMP 1 WT and KO mice over 20 weeks on 45% HFD. **C:** Weekly cumulative food intake (Kcal) of RAMP 1 WT and KO mice over 20 weeks on 45% HFD. **D:** Non-fasting blood glucose level (mmol/l) of RAMP 1 WT and KO mice maintained on 45% HFD for 20 weeks measured on a bi-weekly base. All data are expressed as mean ± SEM, symbols denote significant differences between the two genotypes; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N = 24 per group.

As for the other KO models, the same protocol was repeated in RAMP 1 WT and KO mice. Baseline body weight at the beginning of the HFD period was similar (21-23 g) in both groups. Overall, we didn't observe any significant difference during the HFD period, with exception of week 16, where RAMP 1 WT mice showed a significantly higher body weight compared to the KO mice (Figure 12A).

Similarly to the RAMP 3 KO study, 45% HFD had the same effect on body weight gain in WT and KO mice (Figure 12B). However food intake was significantly higher in RAMP 3 KO mice from 13 to 17 and 20-23 weeks-old (Figure 12C).

No significant difference on blood glucose level was measured between both genotypes (Figure 12D).

### 4.3.2 Effect of amylin-selective agonist NN1213 and sCT in RAMP 1 WT and KO mice models after 21 days

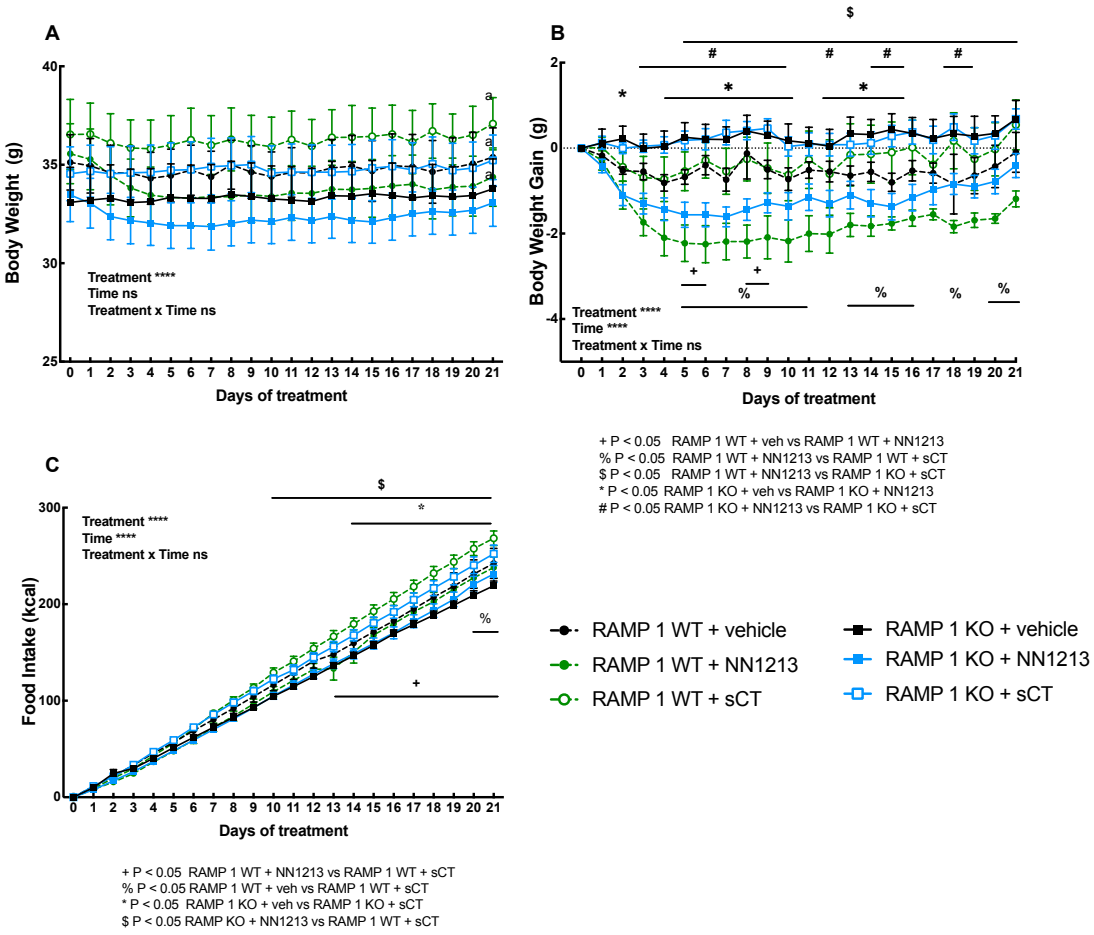


**Figure 13. Effect of amylin-selective agonist NN1213 (30 nmol/kg) and sCT (150 nmol/kg) subchronic treatment SC (21 days) in RAMP 1 WT and KO mice after an induction obesity period of 20 weeks on 45% HFD. A:** daily BW (g) of RAMP 1 WT and KO mice treated for 21 days with vehicle, NN1213 (30 nmol/kg) and sCT (150 nmol/kg). **B:** daily body weight gain (g) of RAMP 1 WT and KO mice treated daily with vehicle, NN1213 and sCT for a period of 21 days. **C:** daily food intake of RAMP 1 WT and KO during 21 days of treatment with vehicle, NN1213 and sCT. Data are represented as mean ± SEM; N=8/group. Parameters with differing superscript letters differ from each other at $p<0.05$ level by Tukey post hoc adjustment after significant intergroup differences were found by two-way ANOVA.

As shown in figure 13A, the six groups had similar baseline body weight at the start of the injection period. During the first week of treatment, RAMP 1 WT and KO treated with NN1213 responded to the treatment and gained less weight compared to their respective vehicle-treated and sCT-treated mice (Figure 13B). In the second and third week of treatment, NN1213-treated RAMP 1 WT and KO mice maintained their reduced body weight loss in comparison to the vehicle control group. While NN1213 seemed to induce a greater decrease in body weight gain in WT mice compared to KO mice, this difference in effect was not significant. sCT had no effect on RAMP 1 WT and KO mice (Figure 13B).

On day 13 of treatment, cumulative food intake of RAMP1 WT receiving sCT was significantly increased compared to WT NN1213 and vehicle-treated mice. This difference remained significant until the end of the treatment period (Figure 13C). No other treatment effects were otherwise observed.

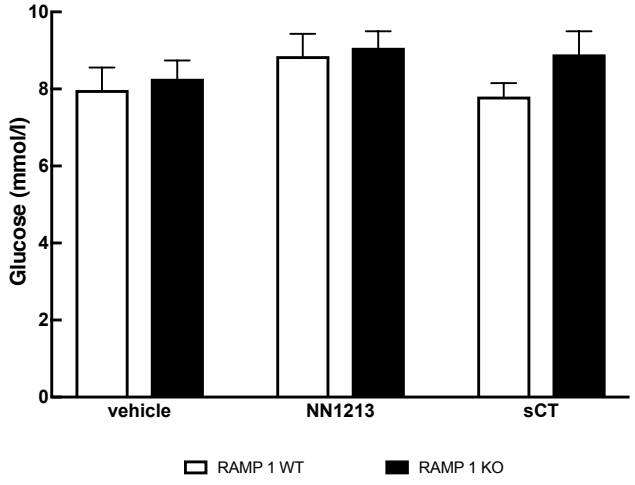


**Figure 14: blood glucose level (mmol/l) at time of sacrifice in RAMP 1 WT and KO mice after been treated daily for 21 days with vehicle, NN1213 (30 nmol/kg) and sCT (150 nmol/kg).** Data are represented as mean ± SEM. N=8/group

No genotype or treatment effect on blood glucose level was observed at sacrifice (Figure 14).

## 4.4. Comparison of the nasal-anal length between RAMP 1 and 3 WT/KO mice after treatment

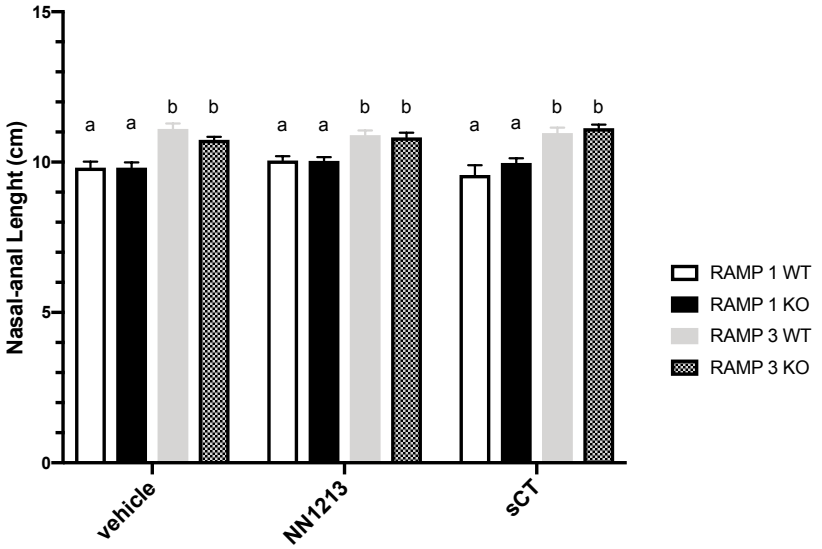


**Figure 15: Nose-Anus length of RAMP 1 WT and KO vs. RAMP 3 WT and KO mice (cm) measured at time of sacrifice** Data are represented as mean ± SEM. N=8/group. Parameters with differing superscript letters differ from each other at $p<0.05$ level by Tukey post hoc adjustment after significant intergroup differences were found by two-way ANOVA.

Since starting body weight was much lower in RAMP 1 WT/KO mice, nasal-anal length of RAMP 1 and RAMP 3 mice at the end of the treatment period was measured. Thus, RAMP 1 WT and KO mice were significantly shorter than RAMP 3 WT and KO mice. No treatment effect was otherwise observed.

## 5. Discussion

The purpose of this study was to assess the weight loss efficacy of the amylin selective agonist (NNCO1741213) in the three RAMP mouse models- RAMP 1/3, RAMP 3, RAMP 1- WT and KO compared to that of sCT to investigate the role of the different RAMPs in modulating the pharmacological effects and potential differences of amylin selective agonist versus dual amylin calcitonin receptor agonist.

Our results showed that RAMP 1 and RAMP 3 are involved in the mechanism of action of NN1213 in mice and both receptor isoforms mediate the action of the amylin selective agonist on body weight and food intake. Indeed, we saw a weight – lowering effect of NN1213 in all WT mice and in the KO mouse models where at least one RAMP (RAMP 1 or RAMP 3) was expressed.

Further, we provided evidence that sCT in mice has an effect on body weight and food intake only in the absence of RAMP 3. In addition to that our results suggest that sCT does not have any effect in RAMP WT mice, which is different from rats[54].

### 5.1 Effect of 20 weeks 45% high fat diet on body weight and food intake in RAMP mice models

In the first part of the study, we investigated the effect of 20 weeks of HFD ad libitum in the three mouse models. Our results show surprisingly that RAMP 1/3 KO mice gained significantly less weight than the WT during 20 weeks of 45% HFD. In the RAMP 3 model, body weight of KO and WT mice was similar for the entire duration of HFD period. Last, in the RAMP 1 model, we noticed that RAMP 1 KO gained less weight from week 14 until the end of the observation period. The same effect was also noticeable in the cumulative food intake in both RAMP 1 and 1/3 KO models with both KO eating less HFD than their WT littermates while RAMP 3 KO mice ate the same amount as WT. Finally, only RAMP 1/3 KO mice had lower blood glucose level than the WT, reflecting the lower body weight and the lower amount of food consumed. These effects remained significant until the end of the entire study.

Opposite to what we have shown in our previous study where RAMP 1/3 KO gained more weight than WT when fed 45% HFD at 8 weeks-old[58], here the mice were put on HFD at 4 weeks of age and presumably had not yet reached puberty at that time. Based on our results, the lower body weight gain and lower food intake on 45% HFD in RAMP 1/3 and RAMP 1

KO mice suggest that these mice may become resistant to a high fat palatable diet regimen when exposed very early in life.

One other consideration to be discussed is that RAMP 1 mice were generally lighter than the other two KO models and our results showed also that RAMP 1 were smaller than RAMP 3 KO mice. These differences could be explained by a breeding effect or by the role RAMP 1 vs. RAMP 3 on growth and body weight homeostasis. It is known that chow diet-fed RAMP 1 overexpressing transgenic mice are leaner, lighter, have increased energy expenditure and higher body temperature[59], but we surprisingly also found out that 45% HFD-fed RAMP 1 KO mice were leaner than the WT mice. It is possible that the germ-line deletion of RAMP 1 induces developmental effects or that the depletion of RAMP 1 in altering other signaling pathway such as CGRP [60, 61] plays a role in this metabolic effect. Our previous study on chow diet showed that even though RAMP 1 KO weigh the same as WT mice, they were fatter than the WT mice suggesting that RAMP 1 mediate fat storage and utilization[58]. To explain the "resistance" of RAMP 1 KO to 45% HFD, further experiments are required to understand the underlying mechanism regulating energy homeostasis in RAMP 1 KO mice.

On chow diet, male RAMP 3 WT and KO are known to display similar body weight and food intake[62]. Similar to our study with male mice, female RAMP 3 WT and KO mice fed 32% HFD also had the same body weight and food intake. These female KO mice also had the same glucose tolerance and body composition compared to WT[62]. However, contrary to our study, Liu *et al* did not assess the effect of HFD in male mice.

## 5.2 Effect of the subchronic NN1213 and sCT treatment in WT/RAMP KO mice models

### 5.2.1 RAMP 1/3

Our results clearly demonstrated that RAMP 1/3 WT mice were sensitive to the NN1213 compound, at least in the first 5 days of treatment. Then we observed a rebound phenomenon and mice regained their body weight by day 15 of treatment. This initial decrease in body weight was accompanied by an anorectic effect during the first week of treatment.

While sCT had a minimal effect in the WT mice, sCT was efficient in the KO mice eliciting a body weight loss in the first 7 days of experiment, while thereafter, as observed for RAMP 1/3 WT receiving NN1213, mice started to regain some weight and reached their initial body weight measured by the end of the treatment period. This temporary decrease in body weight

was also accompanied by an anorectic effect during the first week of treatment. Furthermore, this decrease in body weight gain and food intake was correlated with a decrease in blood glucose and insulin in sCT-treated KO mice compared to the sCT-treated WT mice.
Furthermore, WT and KO mice display significantly different body weight at the start of the experiment with KO mice being lighter than WT mice. This difference in body weight also correlated with glucose levels with WT mice having an overall higher glucose level than RAMP 1/3 KO mice. The body composition analysis confirmed that regardless of the treatment received, RAMP 1/3 KO mice were leaner compared to the WT cohort. Although these differences didn't reach significance, WT mice receiving NN1213 tended to be leaner in comparison to the other WT groups but still heavier than the KO. Interestingly, body fat mass analysis didn't show any differences between WT and KO mice and displayed a similar fat ratio (%), similar subcutaneous fat and there was only a trend (P=0.06) in visceral fat between WT and KO receiving both sCT. Based on these results, we expected no differences in leptin levels between the groups; nevertheless we clearly saw that WT mice treated with NN1213 had significant lower plasma leptin level compared to the other WT groups. Thus even though there was no difference in subcutaneous tissue mass, leptin levels were lowered in WT NN1213-treated mice suggesting that NN1213 may act on leptin production since subcutaneous fat tissue is the major source of leptin in the blood[63]. It is also possible that subcutaneous fat pads were decreased in WT-treated mice but since we only quantified it from L1 to L6 we might have missed this difference between the groups.
In addition, we observed that all KO mice had significant lower amount of plasma leptin level in comparison the WT vehicle-treated group further highlighting their lower body weight on 45% HFD.

Together these data suggest that subchronic sCT only had an effect in the RAMP 1/3 KO mice and that NN1213 elicited body weight loss only in the WT mice. Thus the NN1213 is specific to the amylin receptor while sCT can act when only CTR is present. To understand why subchronic sCT does not elicit any weight loss and does not decrease food intake in WT mice require further investigation.

### 5.2.2 RAMP 3

RAMP 3 WT treated with NN1213 and RAMP 3 KO mice treated with sCT showed a similar trend throughout the entire experiment. Our results showed a strong body weight loss during

the first week of treatment in both groups. Then this effect was partly lost and a rebound phenomenon was observed in both groups. Interestingly, compared to RAMP 1/3 KO, NN1213 seemed to have some effect in the RAMP 3 KO mice, leading to a body weight loss of approximately 2g. Nevertheless, compared to RAMP 3 WT, RAMP 3 KO were less sensitive to NN1213. As already observed in the RAMP 1/3 WT mice, sCT didn't have any effects in the RAMP 3 WT mice. Our findings showed no differences in food intake amongst all the groups. Finally, we did not see any increase in body weight gain in vehicle-treated group and WT treated with sCT, compared to what we have seen in the double KO group. The single RAMP3 KO/WT colony treated with vehicle and sCT maintained their body weight constant whereas the double KO mice (RAMP 1/3 KO) treated with vehicle and sCT gained more weight. We could not find any explanation for this discrepancy.

### 5.2.3 RAMP 1

RAMP 1 WT mice treated with NN1213 showed more weight loss in comparison to the other vehicle and sCT-treated WT mice. WT 1213-treated mice also lost significantly more weight compared to KO receiving sCT. In fact, sCT had no effect neither in the WT nor in the KO mice.

In addition, RAMP 1 KO mice were also responsive to NN1213, although this effect was less pronounced in comparison to the WT receiving NN1213. The response of these mice was significant in comparison to KO mice receiving sCT. Our results showed that the main difference in the RAMP 1 KO/WT mice colony is that, compared to the other two groups, that they were not responsive to the effect of sCT, regardless of the genotype. This result was not present in RAMP 1/3 and RAMP 3 mice colonies, where the KO mice were both sensitive to sCT. This is in accordance to our recently published study where acute sCT did not induce any anorectic effect in the RAMP 1 KO mice[58].

Overall, we also observed that mice receiving sCT ate more than the others, this effect was noticeable especially in the second half of treatment and similar to what was observed in RAMP1/3 KO/WT mice.

## 5.3 Comparison between the three WT and KO mice models

Our findings showed that NN1213 elicited body weight loss in each of the WT models receiving NN1213 at a dose of 30 nmol/kg once daily for 21 days.

The body weight loss was between -2g and -3g for all the WT-treated mice. WT NN1213-treated mice lost most of their weight in the first 5 days of treatment and this weight loss was maintained for the RAMP 1 WT and RAMP 3 WT. However even though the WT mice were issued from the RAMP 3 breeding colony, we observed that RAMP 1/3 WT mice treated with NN1213 lost more weight in the first 5 days of treatment, then, during the rest of the experiment we observed a rebound phenomenon where the animals constantly regained weight and between day 17 and day 18 they reached the initial body weight (day 0). At sacrifice, the animals weight exceeded the initial body weight. A difference in breeding generation might explain this difference between the 2 experiments.
Thus to conclude, when given for a prolonged period NN1213 shows the strongest effect on mice in the first 5-6 days of treatment. Then this effect goes partly lost generating a rebound phenomenon where the animals generally regain some weight.

NN1213 was also able to lower body weight in RAMP 3 and RAMP 1 KO mice, but not in RAMP 1/3 double KO mice. What we noticed was that the compound NN1213 caused more body weight loss in RAMP 3 KO (-2g) animals than RAMP 1 KO (-1g). We may thus conclude that RAMP 1 may be the main sub-unit mediating the action of NN1213.

Conversely, sCT 150 nmol/kg injected subcutaneously daily for a period of 21 days does not induce body weight loss in WT mice. Indeed, in RAMP 1/3 WT mice we noticed that after a first period where the natural DACRA seemed to have an effect (first 3 days of treatment), the WT mice started to regain weight and at the end of the experiment, the body weight of the animals was higher than the one measured at the beginning of the experiment. A similar effect of sCT was observed in the RAMP 1 WT model, but the body weight gain in this group was lower in comparison to that gained in the RAMP 1/3 WT. RAMP 3 WT lost weight in the first 3 days of treatment, then they slightly regained weight, but the body weight of these animals at the end of the experiment was similar to the baseline body weight measured after 20 weeks of HFD. Overall subchronic sCT had little to no effect in WT mice while many studies have shown that acute sCT exerts a strong anorectic effect, here[64] suggesting that the accumulation of sCT at the receptor level may induce a desensitization. On the contrary, sCT had an effect in both RAMP 1/3 double KO and RAMP 3 KO mice but no effect at all in the RAMP 1 KO mice. All together, these results indicate that the absence of RAMP 3 promotes the weight lowering effect of sCT in mice.

Based on these findings we observed two main differences between the action of amylin selective analogues (NN1213) and dual amylin selective agonists (sCT) in mice: an amylin selective agonist can elicit body weight in the presence of one or both RAMP 3 and RAMP 1, showing more effect when both RAMPs are present, while the DACRA sCT can elicit body weight loss only in the absence of RAMP 3, having no effect at all in the WT models and in the RAMP 1 KO model.

## 5.4. Limitations of the study

### 5.4.1 Differences between rats and mice

Several studies have demonstrated that sCT, other DACRA compounds or amylin and its analogs are able to induce long term weight loss in diet induced obese rats[50, 55, 56]. Rats treated s.c. subchronically with one of the above-mentioned peptides loose weight and transiently reduce their food intake in a dose-dependent manner. For example, rats treated for 56 days with 2.5 ug/kg KBP-089 (DACRA) s.c. showed a body weight loss of 17%. Moreover, KBP-089 reduced fat depot size and fat accumulation in liver and in muscles[56]. Other two DACRA have been produced: KBP-088 and KBP-042 which are also able to reduce body weight when injected or given orally in a sustained and dose-dependent manner; additionally they also reduce white adipose tissue mass and adiposity hypertrophy[65, 55].
Unpublished data by Novo Nordisk indicated that diet-induced obese mice respond differently to both amylin and sCT analogues than diet-induced obese rats. Treatment of diet-induced obese mice with amylin analogs induces a minor dose-dependent reduction in body weight during the first 1-3 days of treatment, which is more or less sustained during the remaining treatment period. On the contrary, after 3 days of treatment, animals treated with sCT-based analogues regain or surpass the body weight of vehicle controls in a dose-responsive manner. The results in our study suggest that RAMP WT mice are not sensitive to sCT and that sCT does only elicit body weight loss in the absence of RAMP 3, suggesting a role of RAMP3 in the resistance to sCT action in mice. In mice, it is not known how the amylin receptors are expressed and how they exactly work. Indeed it is still unknown which RAMPs are expressed in which brain area, how they interact with CTR and with which RAMPs these DACRA mostly interact with and how is the signaling pathway regulated. The assumption has been that mouse and rat amylin receptor behave similarly, but this may not be true.

While the present study has shed some light on the specific role of RAMP in mediating the effect of DACRA and amylin selective analogue, further studies need to be conducted to investigate the role and the exact expression of AMY1-3 in mice to properly understand their mechanism of action in mouse models.

### 5.4.2 Role of RAMP 2

The role of RAMP 2 combined with CTR composing AMY2 is not well investigated. Unlike RAMP 1 and RAMP 3 KO mouse models which survive during embryonic development and in the adulthood, RAMP 2 KO mice are embryonically lethal, showing generalized edema[66]. Several studies demonstrated that RAMP 2 is essential for angiogenesis and for the development and integrity of a functional lymphatic system[67, 68, 69]. Knocking out the gene responsible for the expression of RAMP 2 causes lymphatic and blood vessels defects.
These findings indicate that endogenous expression of RAMP 1 and RAMP 3 are not able to compensate for the physiological function of RAMP 2[66]. In contrast, global deletion of either RAMP 1 or RAMP 3 does not affect survival, perhaps because the expression of other RAMPs and particularly RAMP2 is able to compensate for their absence and their loss of function[66]. Consequently, no global RAMP 2 KO mouse model can be generated to investigate the role of RAMP 2. This limits the characterization of this protein in modulating the expression of amylin receptor and in the pharmacological role of AMY2 in the control of food intake and body weight when interacting with amylin, amylin agonists and dual amylin and calcitonin receptor agonist.

### 5.4.3 Overexpression of RAMPs when one knocked out

If one RAMP is knocked out, we may expect the other two to be overexpressed. Based on this concept, we may expect that RAMP 3 KO mice showed an overexpression of RAMP 1 and RAMP 2 trying to compensate the loss of function of RAMP 3. Unfortunately, we don't know if RAMP 2 was elevated on our KO models. What we observed was that RAMP 3 and 1 WT and KO mice had similar body weight for the entire duration of the experiment suggesting that the depletion of only one RAMP does not affect body weight. On the contrary, previous studies[59,70,66] demonstrated that RAMP 1 overexpression in mice leads to lower body weight and increased energy expenditure; transgenic mice that overexpressed

RAMP 1 mice are leaner and lighter in comparison to the WT. Previous studies also demonstrated that mice overexpressing RAMP 2 exhibited differences only in the vascular system[66]. Further analysis about the expression pathways, compensatory mechanisms and expression sites of RAMPs will provide more information and definitive answers[68].
Overexpression of RAMP 3 has not been investigated yet.

In summary, our findings suggest that in mice RAMP 1 and RAMP 3 partly mediate the effect of an amylin selective agonist and that a dual amylin-calcitonin receptor agonist is able to elicit body weight loss and to control food intake only in the absence of RAMP 3.

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